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Construction and validation of metagenomic DNA libraries from landfarm soil microorganisms

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ABSTRACT. Landfarming biodegradation is a strategy used by the petrochemical industry to reduce pollutants in petroleum-contaminated soil. We constructed 2 metagenomic libraries from landfarming soil in order to determine the pathway used for mineralization of benzene and to examine protein expression of the bacteria in these soils. The DNA of landfarm soil, collected from Ilhéus, BA, Brazil, was extracted and a metagenomic library was constructed with the Copy Control™ Fosmid Library Production Kit, which clones 25-45-kb DNA fragments. The clones were selected for their ability to express enzymes capable of cleaving aromatic compounds. These clones were grown in Luria-Bertani broth plus L-arabinose, benzene, and

chloramphenicol as induction substances; they were tested for activity in the catechol cleavage pathway, an intermediate step in benzene degradation. Nine clones were positive for ortho-cleavage and one was positive for meta-cleavage. Protein band patterns determined by SDS-polyacrylamide gel electrophoresis differed in bacteria grown on induced versus non-induced media (Luria-Bertani broth). We concluded that the DNA of landfarm soil is an important source of genes involved in mineralization of xenobiotic compounds, which are common in gasoline and oil spills. Metagenomic library allows identification of non-culturable microorganisms that have potential in the bioremediation of contaminated sites.

Key words: Benzene; Toluene; Xilene; Soil; Metagenomic library; Biodegradation

INTRODUCTION

Tonnes of wastes of several kinds are produced by industries and accumulated in the environment. These residues have chemical compositions and molecular structures that are not recognized by degradative enzymes, thus resisting biodegradation, or they are not completely metabolized (Pereira Netto, 2000). Aromatic hydrocarbons such as benzene, toluene, and xylene are considered recalcitrant due to their difficult degradation because of complex structures with a ring structure and resonance in their chemical bonds (Smith, 1994). The generation of these anthropogenic compounds, through oil-related production, introduces into the environment each year large amounts of aromatic hydrocarbons, resulting in the contamination of ecosystems (Jacques et al., 2007).

Studies have shown that microorganisms can break down aromatic rings, such as those of benzene, toluene, and xylene, and mineralize their carbon skeleton (Bamforth and Singleton, 2005). The catabolism of the benzene ring occurs through oxidation of the ring to a dihydroxy-aromatic compound (a catechol or hydroquinone), followed by oxidative cleavage of the ring (Bugg and Winfield, 1998).

"Landfarming" is a method used by petrochemical industries to prevent the release of waste from refineries into the environment. It is a natural source of microorganisms capable of degrading petroleum-derived by-products (Maciel et al., 2007). Current attempts to describe and understand microbial diversity are designed to overcome the bias of cultivation, providing a more accurate picture of diversity and their function in the natural environment. Thus, metagenomics is a tool that eliminates cultivation steps, as it consists of direct extraction of environmental DNA and its cloning in an appropriate vector (Handelsman, 2004).

The 2 most commonly used vectors for metagenomic library construction are fosmid and bacterial artificial chromosome vectors (Handelsman, 2004). The fosmid vector has an affinity for fragments of 25-45 kb. Thus, a fosmid library has a lower percentage of clones without inserts or small inserts (Rondon et al., 2000). In this study, we constructed and validated 2 metagenomic libraries with DNA of landfarm soil using a fosmid vector, aiming at the biodegradation of aromatic hydrocarbons.

MATERIAL AND METHODS

Soil samples

Landfarm soil contaminated with petroleum waste from Landulpho Alves Refinery, Camaçari, BA, was sampled from the surface to a depth of 10 cm (Maciel et al., 2009). Soil 1 (Landfarm - LF) consisted of crude oil-contaminated soil. Soil 2 (Landfarm - LF^+) was continuously enriched for 9 months with petroleum and a minimal medium.

DNA extraction and library construction

High molecular weight DNA from landfarm soil was obtained by extraction with the PowerMax® Soil DNA Isolation kit (MoBio), and the metagenomic library was constructed with the Copy Control™ Fosmid Library Production Kit (EPICENTRE®) according to manufacturer instructions. DNA was purified by electrophoresis on 1% low melting temperature agarose. DNA larger than 30 kb was excised and extracted from the gel with GELase (Epicentre) according to manufacturer instructions. This was ligated to the Copy Control pCC2FOS™ vector (EPICENTRE®), with minor modifications. The vector insert ligation incorporated lambda phage (MaxPlax Lambda Packaging Extracts) competent cells that will infect *E. coli* EPI300-$T1^R$ according to manufacturer instructions (EPICENTRE®). The reaction mixture was added to Luria-Bertani broth with 12.5 µg/mL chloramphenicol, and grown cells were collected and stored at -80°C.

Test for cleavage of catechol

The clones were grown in Luria-Bertani broth plus L-arabinose, benzene, and chloramphenicol for induction. The cleavage of catechol, an intermediate in the benzene degradation pathway, was determined by incubating grown cells in a solution containing Tris-HCl, pH 8.0, catechol, and toluene. The meta-cleavage of the substrate was observed by the formation of a yellow color due to accumulation of 2-hydroxymuconate semialdehyde. When the yellow color was not observed, the cells were incubated and tested for the presence of β-ketoadipate detected by the Rothera reaction. The purple color indicates the breakdown of catechol through the ortho-cleavage pathway (Hammann and Kutzner, 1998).

SDS-PAGE

The difference in the expression pattern of proteins involved in the cleavage of the aromatic ring was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of extracts of cells grown in induction medium (Luria-Bertani broth plus L-arabinose, benzene, and 12.5 µg/mL chloramphenicol) and non-induction medium (Luria-Bertani broth plus 12.5 µg/mL chloramphenicol). Proteins were extracted from pelleted cells after resuspension in lysis buffer, addition of lysozyme, and sonication.

RESULTS AND DISCUSSION

Both libraries were screened for cleavage of the aromatic ring of catechol by catechol

2,3-dioxygenase as well as catechol 1,2-dioxygenase. Accordingly, the LF library had 3360 clones and the LF^+ library, 4416 clones. Nine clones were obtained for ortho-cleavage of catechol, namely 1 LF and 8 LF^+ clones of the libraries.

The positive control used was a bacterial isolate from landfarm soil (Maciel et al., 2007). In the ortho-cleavage pathway, aromatic hydrocarbons are oxidized by dioxygenases to cis-dihydrodiol, and this is converted to catechol. The dihydroxyaromatic ring is then opened by the oxidative ortho-cleavage enzyme catechol 1,2-dioxygenase and cofactor Fe^{3+}, resulting in cis, cis-muconic acid. This is cyclized enzymatically to form a 5-membered lactone, muconolactone. An isomerase enzyme then transforms it into the unsaturated lactone, which is hydrolytically cleaved to give β-ketoadipate. This acid is subsequently cleaved to give acetic acid and succinic acid, which can be utilized for growth via the tricarboxylic acid cycle. The reaction is characterized by a purple color due to the presence of β-ketoadipate (Figure 1) (Bugg and Winfield, 1998).

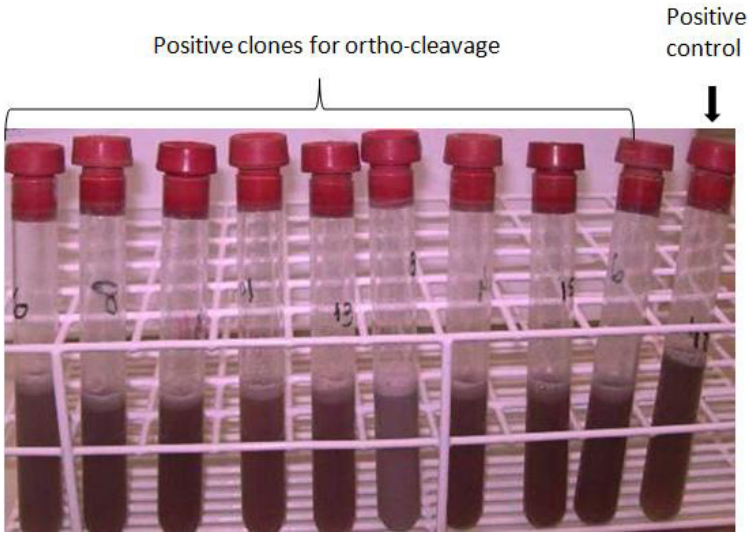

Figure 1. Nine positive clones for ortho-cleavage and a positive control characterized by purple color after a 12-h incubation at 37°C by the reaction of Rothera.

The meta-cleavage pathway is characterized by a yellow color due to accumulation of 2-hydroxymuconate semialdehyde (Figure 2). One positive clone for this pathway was found in the LF^+ library. Catechol is opened by catechol 2,3-dioxygenase and cofactor Fe^{2+}, resulting in hydroxymuconate semialdehyde. The ring fission product 2-hydroxymuconaldehyde acid can be oxidized to give 2-hydroxymuconic acid, which is decarboxylated through the combined action of a tautomerase enzyme and a decarboxylase enzyme to give 2-hydroxypentadienoic acid. This is further broken down by hydration to 4-hydroxy-2-oxopentanoic acid, followed by aldolase-catalyzed cleavage to give acetaldehyde and pyruvic acid (Bugg and Winfield, 1998). The positive control used was the bacterial strain *Achromobacter xylosoxidans* MCM1, which efficiently metabolizes dibenzothiophene as a source of sulfur (Eixarch and Constantí, 2010).

Figure 2. One positive clone for meta-cleavage and a bacterium strain 59 as the positive control characterized by yellow color after a 15-60-min incubation at 37°C.

The ortho-cleavage pathway occurred more frequently among the clones, probably because it is simpler, having few intermediate compounds, or because it involves fewer enzymes and genes, making it easier to obtain complete operons in this pathway. These results suggest that the DNA of landfarm soil is an important source of new genes involved in the mineralization of xenobiotic compounds.

The results showed that the number of bacteria able to grow in medium contaminated with benzene was higher after enrichment with oil in the sediment. Thus, it was observed that the presence of oil caused a selection of bacteria capable of degrading aromatic compounds in sediments.

Pieper et al. (2005) described the use the ortho-cleavage pathway in 2 bacterial strains *Arthrobacter protophormiae* RKJ100 and *Burkholderia cepacia* RKJ200, in pesticide degra-

dation. Arenghi et al. (2001) reported on the mechanism of regulation of meta-cleavage by *Pseudomonas stutzeri* OX1. Furthermore, Murray and Williams (1974) reported that *P. putida* NCIB 10015 metabolizes phenol and cresols by meta-cleavage and benzoate by the ortho-cleavage pathway, where depending on the growth substrate, the meta- or ortho-pathway is stimulated in this bacterial strain. Thus, these authors showed that a bacterial strain can display either metabolic pathway, depending on the available substrate for growth.

Many microorganisms have pathways for the use of aromatic compounds as their sole source of carbon and energy (Harwood and Parales, 1996; Pieper, 2005). Metabolic pathways and associated genes for the cleavage of the aromatic ring have been characterized in many strains of *Proteobacteria* and *Actinobacteria.* In the case of pollutants such as benzene, toluene, naphthalene, biphenyl, or polycyclic aromatic, aerobic degradation is initiated by activation of the aromatic ring through reactions catalyzed by iron-dependent oxygenases (Gibson and Parales, 2000).

Genes involved in the degradation pathways of aromatic hydrocarbons have been reported in many catabolic operons, which are predominantly found in areas contaminated with benzene (Junca et al., 2004). Functional screening done with metagenomic libraries has led to the discovery of novel genes encoding antibiotic resistance (Riesenfeld et al., 2004), esters and glycosyl hydrolases (Ferrer et al., 2005), and polyphenol oxidases (Beloqui et al., 2006) and has also provided information about the diversity of estradiol dioxygenases found in wastewater plants (Suenaga et al., 2007).

Estradiol dioxygenases are key enzymes in degrading aromatic compounds and many of these proteins and their coding sequences have been described, purified, and characterized in recent decades (Brennerova et al., 2009). Catechol 2,3-dioxygenases are a group that display significant activity against catechol (Eltis and Bolin, 1996). Substrate-dependent microorganism cultivation has shown a wide diversity of catabolic genes expressing estradiol dioxygenase activity. The positive clone for catechol meta-cleavage was grown in LB medium, induced and non-induced (Figure 3), and demonstrated an increased expression of proteins with molecular weights of 35-45 kDa compared to clones grown under induction conditions.

The differential expression of proteins yielded positive clones for ortho-cleavage, which were evaluated under the same conditions previously described (Figure 4). Nine clones were obtained, of which 7 (1I, 2I, 3I, 4I, 6I, 7I, and 9I) showed expression of proteins with molecular weights of 35-45 kDa.

Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, responsible for ortho- and meta-cleavage, respectively, have molecular weights of 30-40 kDa (Vetting and Ohlendorf, 2000). They are members of the oxidoreductase family, which insert oxygen atoms in the molecule, promoting cleavage of the aromatic ring in the ortho-position, forming cis,-cis-muconic acid or 2-hydroxymuconate-semialdehyde if cleavage occurs at the meta-position.

The information obtained from this study makes it possible to evaluate the potential of the metagenomic library of landfarm soil. Non-culturable microorganisms can be used for the biodegradation of aromatic hydrocarbons, which are present in oil products and are difficult to degrade components, resulting in pollution of the environment where they exist in large quantities.

This study confirmed the effectiveness of using metagenomics as a tool to investigate non-culturable microorganisms and their potential in bioremediation of contaminated sites. Furthermore, the effectiveness of high molecular weight DNA cloning is very important for the discovery of novel enzymes of great industrial interest, since the study of these DNA sequences in large fragments increases the chance of these discoveries.

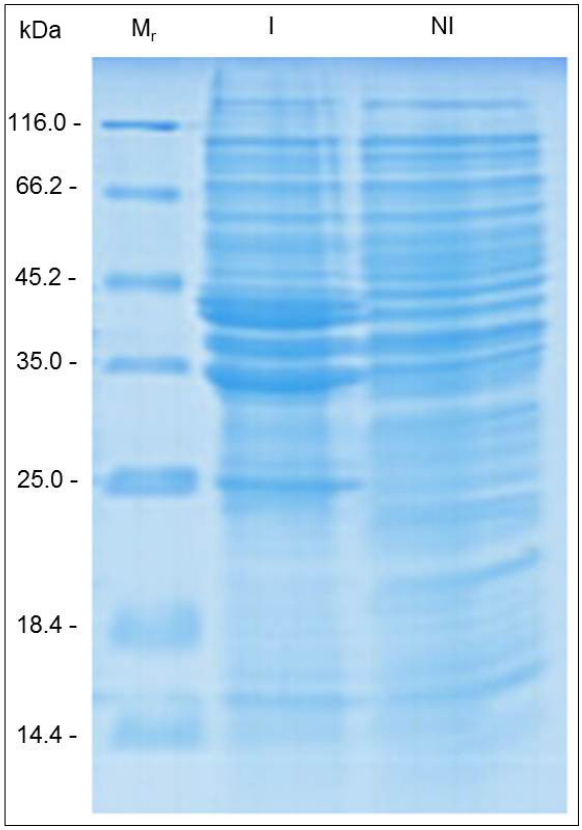

Figure 3. Band profile of proteins extracted from the positive clone target for cleavage. M_r = marker-fermentas; I = induced medium; NI = non-induced medium.

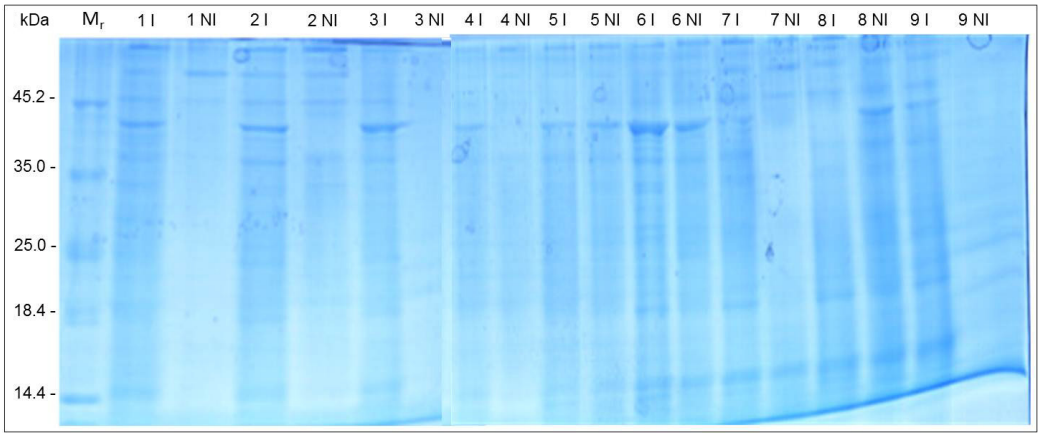

Figure 4. Band profile of proteins extracted from the positive ortho-cleavage clones. For abbreviations, see legend to Figure 3.

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